Genetic variation in matrix metalloproteinase *MMP2* and *MMP9* as a risk factor for idiopathic recurrent spontaneous abortions in an Indian population

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Abstract

Purpose Matrix metalloproteinases, *MMP2* and *MMP9*, are found to have an important role during ovulation and pregnancy because of their capacity to degrade components of the extracellular matrix (ECM) thereby facilitating cell migration and angiogenesis. In this respect, the aim of the present study was to evaluate the association of the promoter polymorphisms –1306 C > T and –1562 C/T in *MMP2* and *MMP9* respectively with couples diagnosed with idiopathic recurrent spontaneous abortions (IRSA). The expression levels of these two genes were also studied in fetal tissue.

Methods In this case control study, a total of 35 couples with at least three consecutive IRSA and 35 fertile couples were included. Genotype analysis was performed using polymerase chain reaction and Sanger sequencing.

Results No statistically significant differences were found in distribution of *MMP2*-1306C/T and *MMP9*-1562C/T genotypes in the three groups between the cases and controls.

Conclusion Further genetic association studies on a larger number of IRSA couples, as well as evaluation of more MMP polymorphisms and their expression profiling are needed to establish the potential role of MMP polymorphisms in IRSA.

Keywords Idiopathic recurrent spontaneous abortion · Matrix metalloproteinase · Single nucleotide polymorphism · Fetal genotypes

Introduction

Matrix metalloproteinases (MMP) are a family of zinc and calcium-dependent endopeptidases which play a significant role in various biological processes like tissue remodeling, healing, and reproduction. Of the 25 human MMPs [1–5] that have been identified, *MMP2* and *MMP9*, also termed as gelatinases, are found to have an important role during ovulation and pregnancy because of their capacity to degrade components of the extracellular matrix (ECM) thereby facilitating cell migration and angiogenesis [6]. During placentation, *MMP2* and *MMP-9* breakdown type IV collagen of the uterine endometrium increasing the invasiveness of the trophoblastic cells, which is needed for the development of maternal-fetal interface, and also influences fetal development by means of vasculogenesis [6–11]. An orchestrated action of *MMP2* and *MMP9* along with their inhibitors is required for a successful pregnancy outcome wherein any dysregulation in either *MMP2* or *MMP9* function could result in abnormal tissue remodeling and eventually pregnancy loss [6].

Idiopathic recurrent spontaneous abortions (IRSA), defined as three or more consecutive pregnancy losses before the 24th week of gestation, affects approximately 3% of couples trying to conceive [7]. In the mid-secretory phase of the endometrium of women with recurrent miscarriages, higher levels of *MMP2* and lower levels of *MMP9* expression were observed when compared with women who had uneventful pregnancies [8–10]. This altered expression, in part, could be attributed to single nucleotide

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polymorphisms (SNP) which are common genetic variations found in the human genome that could sometimes lead to the improper functioning of the gene. There is strong evidence that the expression level of *MMP2* and *MMP9* are affected by SNP in the promoter region of the genes which decreases transcriptional activity. In this respect, the aim of the present study was to evaluate the association of the promoter polymorphism –1306 C > T and –1562 C/T in *MMP2* and *MMP9* respectively with couples diagnosed with IRSA. The expression levels of these two genes were also studied in the fetal tissue.

Materials and methods

Subjects

A total of 35 couples with at least three or more recurrent pregnancy losses and 35 fertile couples were included in the study as cases and controls, respectively. A questionnaire was administered to all subjects to collect demographic characteristics, lifestyle (consumption of alcohol, coffee, tobacco, and/or drug use), and family history data (Table 1). For inclusion in the study, all of the following causes of IRSA were eliminated: abnormal chromosomes in either of the partner by standard G banding, antiphospholipid antibodies using the lupus anticoagulant test, uterine anomalies by ultrasound imaging, endocrine dysfunction, and maternal infections by assessing microbiological cultures of the samples obtained from the cervix and uterine cavity. The couples in the control group also had normal karyotypes, no history of pregnancy loss, no endocrine disorders, and had at least one successful pregnancy. The selected couples in the control group were undergoing elective termination of the pregnancy for mainly socio-economic reasons. All participants were Indian and recruited from Sri Ramachandra Medical College in Chennai after obtaining an informed consent. The placental tissue obtained from both the cases and controls were screened for chromosomal abnormalities by standard

karyotyping procedures [11], and only chromosomally normal specimens were taken for the genotyping.

Genotyping

High molecular weight genomic DNA was extracted from whole blood by salting-out method [13] and using the QIAGEN miniprep DNA isolation kit for the tissue specimens. Polymerase chain reaction (PCR) was performed to amplify the promoter sequences of *MMP2* and *MMP9* gene using the following primers: *MMP2*-1306 C/T: forward 5'-CTT CCT AGG CTG GTC CTT ACT 3' and reverse 5'-CTG AGA CCT GAA GAG CTA AAG 3'; *MMP9*-1536C/T: forward 5'-GCC TGG CAC ATA GTA GTA GGC CC 3' and reverse 5'-CTT CTT AGC CAG CCG GCA TA 3'. Using the ABI Prism Big Dye Terminator V.1.1, sequencing of the amplicons was performed according to the manufacturer's instructions (ABI, Foster City, CA, USA), and SNP genotyping was performed on an ABI 3730 automated sequencer.

Expression analysis

From the placental tissue samples total ribonucleic acid (RNA) was isolated using the TRIzol (Merck) method. After quantification using a nanodrop, the RNA was converted to cDNA using a high capacity cDNA conversion kit (Applied Biosystems). Real-time PCR was performed in the Applied Biosystems 9700 HT using SYBR Green chemistry with previously described primers [14] and β -actin as an endogenous control under the program parameters of 95 °C for 10 min, 60 °C for 20 s and data collection at 72 °C. The amount of target gene expressed was calculated using the delta Ct method.

Statistical analysis

To test if the population followed Hardy-Weinberg equilibrium (HWE), the expected genotype and allele frequencies were calculated for the cases and controls. The significance level between the cases and controls was determined by

Table 1 Demographic and clinical characteristics of the study population

Variable	Cases (n = 35 couples)	Control s (n = 35 couples)	<i>p</i> ^a
BMI > 25 kg/m ² , n (%)	7 (20)	6 (17.1)	0.7588
No consumption of tobacco/alcohol/drugs, n (%)	35 (100)	35 (100)	1.0000
Hypertension, n (%)	1 (2.8)	0	0.4946
Dyslipidemia, n (%)	1 (2.8)	1 (2.8)	1.0000
Familial history of cardiovascular disease, n (%)	7 (20)	4 (11.4)	0.3550
Family history of obstetric complications, n (%)	2 (5.7)	1 (2.8)	0.5626

^a The odds ratio (OR), its standard error, and 95% confidence interval are calculated according to Altman and Bland [12]

Table 2 Characteristics of couples with and without IRSA

	Cases (<i>n</i> = 35 couples)	Control (<i>n</i> = 35 couples)
Age in years—median (range)		
Women	26 (20–32)	29 (22–36)
Men	30 (25–35)	34 (25–43)
Parity		
0	35	0
1	0	6
2	0	29
Number of spontaneous abortions		
3	28	0
4	2	0
5	5	0
10	0	0
Duration of gestation of spontaneous abortions/medical terminations		
1st trimester	25	30
2nd trimester	10	5

calculating the odds ratio, confidence interval, and *p* value. In addition, student's *t* test was performed to calculate the difference in expression levels of the two selected genes in the case and control tissue specimens. For all the statistical analysis, a *p* value of <0.05 was considered to be statistically significant.

Results

The mean age and characteristics of the study population are presented in Table 2. The average gestational age of spontaneous and elective abortuses was 12.68 ± 2.9 weeks and 12.5 ± 2.8 weeks, respectively. The difference between the two groups was calculated using the chi-squared test, and the result was not significant at ($p < .05$). There were no

significant differences in subject details, such as body mass index (BMI) and family history, between the cases and controls.

For the *MMP2*-1306 C/T polymorphism the genotype distribution in cases and controls was found to deviate from the HWE. In the case group, while the men showed 94% CC homozygous wild type, 4% CT heterozygous, and 4% TT homozygous mutant genotype, the women had 96% CC homozygous wild type and 4% CT heterozygous, whereas the tissue showed only CC homozygous wild-type genotype. All the three in the control group showed homozygous CC wild-type genotype. Thus, the frequency of the CC genotype was found to predominate in both groups, and there were no significant differences in genotype frequencies between the cases and controls. For the *MMP9*-1562 C/T polymorphism, the distribution of genotype frequency of both cases and controls was in accordance with HWE. A comparison of genotype frequencies showed no difference for the SNP between cases and controls. The genotype frequencies and odds ratio for *MMP9*-1562 C/T are summarized in Table 3.

For gene expression analysis of *MMP2*-1306 C/T, the average delta Ct values for the tissue samples were found to be 7.1 ± 2.7 and 8.1 ± 1.4 , and for *MMP9*-1562 C/T, it was 3.1 ± 1.7 and 4.4 ± 2.7 in cases and controls, respectively. While the fold change in gene expression of *MMP2* in cases compared with controls was increased 1.01-fold, *MMP9* showed a 1.2-fold increase. However, the student's *t* test confirmed that this marginal change for both the genes was not significant.

Discussion

A successful implantation is largely dependent on the degradation of the uterine epithelium and the subsequent invasion

Table 3 Analysis of *MMP9*-1562C/T genotypes

Groups	Genotypes	Controls (<i>n</i> = 35)	Cases (<i>n</i> = 35)	OR	CI (95%)	<i>p</i>
Men	CC	21	16	1.0	—	—
	CT	12	18	1.96	0.74–5.23	NS
	TT	2	1	0.65	0.054–7.89	NS
	CC vs CT + TT	14	19	1.78	0.69–4.59	NS
Women	CC	21	20	1.0	—	—
	CT	12	13	1.13	0.42–3.07	NS
	TT	2	2	1.05	0.13–8.18	NS
	CC vs CT + TT	14	15	1.13	0.43–2.91	NS
Fetus	CC	19	21	1	—	—
	CT	15	11	0.66	0.74–5.23	NS
	TT	1	3	0.65	0.054–7.89	NS
	CC vs CT + TT	16	14	0.79	0.31–2.04	NS

OR odds ratio, CI confidence interval, NS non-significant

of the uterine stroma. Both these physiological processes are closely associated to the expression of MMPs. The trophoblast cells express gelatinases which are considered as crucial enzymes in the trophoblastic invasion process, because they degrade collagen IV of the basement membrane [15]. Previous studies have indicated that either *MMP-9* [15–17] or *MMP-2* [18–20] is significantly expressed during early invasion of the trophoblast. In addition, Xu et al. [20] reported a difference in the expression of *MMP-2* and *MMP-9* in first trimester trophoblast cells, where *MMP-2* was the primary gelatinase secreted until 9 weeks and thereafter *MMP-9*.

This study investigated the association of *MMP2* and *MMP9* functional gene polymorphisms in couples with IRSA, since it has been previously demonstrated that aberrant *MMP* expression was observed in the endometrium of IRSA women and chorionic villi of IRSA concepti [21]. Our findings revealed that for *MMP2*-1306 C/T and the homozygous wild-type genotype was observed almost exclusively in all the groups of both cases and controls. Lower levels of *MMP2* gene expression have been observed in chorionic villi of IRSA concepti [22] indicating that embryonic factors may play a role in placentation. This fact is emphasized by the fact that placental and subsequent fetal growth and development is regulated by both maternal and paternal genomes, and the potential transmission of risk genotypes to the embryo might lead to abnormal ECM remodeling due to lowered *MMP* gene expression. Thus, though our results for the *MMP2*-1306 C/T were not significant, the presence of one mutant homozygous genotype in the IRSA men group confers the possibility that a larger population study may confirm the hypothesis.

For the *MMP9*-1562 C/T SNP, the CC wild-type genotype predominated. This T allele in the promoter region of *MMP-9* gene is believed to cause an increase in *MMP-9* activity, while the CC genotype has been reported to reduce transcriptional activity as a result of inadequate binding of a nuclear repressor protein [23]. As confirmed by in vitro studies, the dysregulation of *MMP9* prevents invasion of cytotrophoblasts and results in embryonic death due to an insufficient maternal–fetal circulation [24]. However, since no significant difference was found in the genotype frequency between the cases and controls, it appears that even though there may be a decrease in the transcriptional activity in the carriers of the CC genotype, it may not be clinically significant to IRSA.

In conclusion, although the results of this study indicate a lack of association between *MMP2*-1306 C/T and *MMP-9*-1562 C/T gene polymorphisms and IRSA, further studies are needed to evaluate the role of these, as well as other *MMP* gene polymorphisms in different populations.

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